ASSESSMENT OF FACTOR VIII GENE IN HAEMOPHILIA A PATIENTS IN EAST COAST MALAYSIA

LAPORAN AKHIR GERAN JANGKA PENDEK
NOMBOR GERAN: 304/PPSP/6131137

PENYELIDIK UTAMA:
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CANSELORI
UNIVERSITI SAINS MALAYSIA

1893

Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik: Dr. RAPIAAH MUSTAFFA

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2) Pusat Pengajian/Pusat/Unit : JABATAN HEMATOLOGI, PPSP, USM

3) Tajuk Projek: ASSESSMENT OF FACTOR VIII GENE REARRANGEMENT IN HAEMOPHILIA A PATIENT IN EAST COAST MALAYSIA

27/4/04

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak:

Bahasa Malaysia	Bahasa Inggeris
Faktor VIII	Factor VIII
PCR panjang	Long PCR
Xba I polymorphism	Xba I polymorphism
Haemophilia A	Haemophilia A

5) Output Dan Faedah Projek

(a) Penerbitan *(termasuk laporan/kertas seminar)*
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan di mana telah diterbit/dibentangkan).

Dibentang semasa research presentation di PPSP pada 28/11/02.

4) (a) Penemuan Projek/Abstrak

*(Perlu disediakan makluman di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan Bahasa Inggeris** Ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti).*

A study was initiated to amplify by PCR, the Xba I polymorphic site in detecting inversion intron 22 in the factor VIII gene. 26 haemophilia A patients were studied from January 2000 to Jun 2002. Inversion mutation of intron 22 were detected in 14.3 % of severe haemophilia A patients. The mutation were not detected in moderate & mild form of haemophilia patients. The inversion mutation of int 22 can be used as one of the mutat for carrier detection & confirmati diagnosis in haemophilia A patien

(Lihat lampiran)

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(b) Faedah-Faedah Lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten.
(Jika ada dan jika perlu, sila gunakan kertas berasingan)

Tiada

(c) Latihan Gunatenaga . Manusia

i) Pelajar Siswazah Tiada

ii) Pelajar Prasiswazah: Tiada

iii) Lain-Lain : Tiada

6. Peralatan Yang Telah Dibeli:

Tiada

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

noted

Professor Zabidi Azhar Mohd. Hussin
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TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENYELIDIKAN

Laporan Kajian

Title

Assessment of Factor VIII Gene in Haemophilia A Patients in East Coast Malaysia

Summary

A study was initiated to amplify by polymerase chain reaction (PCR), the Xba 1 polymorphic site in detecting inversion intron 22 in the factor VIII gene. 26 haemophilia A patients were studied from January 2000 to Jun 2002. Inversion mutation of intron 22 were detected in 14.3% of severe haemophilia A patients. The mutation were not detected in moderate and mild form of haemophilia A patients. The inversion mutation of intron 22 can be used as one of the mutation for carrier detection & confirmative diagnosis in haemophilia A patient.

Introduction

Haemophilia A is X-linked bleeding disorder caused by a deficiency or functional abnormality of coagulation factor VIII, affecting approximately 1 in 10,000 males world-wide and has a severity ranging from mild to severe. 30% of patients have no family history and presumably result from spontaneous mutation.

The gene for factor VIII:C has been assigned to the long arm of human X chromosome at Xq24-qter. Characterization of causative mutations is complicated by the size (186 kb) and organization (26 exons) of the FVIII gene and the heterogeneity of mutations within it[1]. The common factor VIII gene rearrangement is intron 22 inversion found in about 45% of severe Haemophilia A[2]. Genetic analysis of the human factor VIII gene has resulted in accurate carrier detection, confirmative diagnosis of patients and prenatal diagnosis. However, the prevalence involvement and polymorphic variations differs markedly among the ethnic groups. Up to date no Malaysia data has been published.

This study is considerable benefit for accurate carrier detections, confirmative diagnosis and for good counselling in families with haemophilia A and provide insights into the relationships between genetic defects and clinical manifestations.

Objectives

1. To detect the intron 22 inversion in human factor VIII gene of haemophilia A patients.
2. To initiates a preliminary work for family/carrier screening in haemophilia A patients.
3. To develop and establish the molecular method for detection of the above mutation.

Materials and Methods

Patients
Patients consisted of all haemophilia A patients on follow-up at clinics or admitted in the wards in Hospital Universiti Sains Malaysia, Hospital Kota Bharu and Hospital Kuala Terengganu. They were diagnosed as haemophilia A patients by routine coagulation tests such as PT (prothrombin time), APTT (activated partial thromboplastin time), factor VIII clotting activity (FVIII : C) and vWF : RiCof. A total of 10ml blood was collected in the EDTA container from each patient for DNA extraction and further genetic analysis. The other 5ml blood was collected in Sodium trisitrate for coagulation studies.

DNA samples
DNA was extracted from the above samples using standard method established in the human Genetic Unit, School of Medical Sciences, USM.

Amplification reaction
PCR was done in 25 µl total volume containing 200ng genomic DNA, 2.5 µl of Buffer 2 (Expand PCR system, Boehringer Mannheim, Mannheim, Germany), 7.5% DMSO (dimethyl sulphoxide), 250 micro mol/l each of dGTP and 7-deaza-dGTP (Boehringer Mannheim, Mannheim, Germany), 500 micro mol/l from other dNTPs, 1 µl of Expand Long Template DNA polymerase, 0.4 micro mol/l of each primer.

Forward primer (F): GCC CTG CCT GTC CAT TAC ACT GAT GAC ATT ATG CTG AC.
Reverse primer (R): TTC AAC ACG ACC ACC ATC TCT CAA GTG GCC.
F is the primer P as reported by Liu et al (1998)[3,4] and R was designed for specific amplification of the Xbal variable site in int22h-1.

PCR cycling was performed in a GeneAmp2400 thermocycler (from Perkin Elmer), using thin-wall tubes, as follows: 94 degrees C for 2 min followed by 30 cycles of 94 degrees C for 12 s, 65 degrees C for 30 s, 60 degrees C for 7 min with auto-extension of 20 s for the last 20 cycles, followed by 15 min at 68 degrees C. About 5 µl of PCR product was run on 1% agarose gel to check for the presence of the desired product.

Xba1 digestion and electrophoresis. Digestion was done in a total volume of 12 µl containing: 10 µl of PCR product, 10 units of Xba 1 enzyme (from Promega) and 1.2 µl of Xba 1 restriction buffer (buffer D). The digestion reaction was kept at 370C overnight. The digestion product was run on a 20 cm 1% agarose gel at 100 V for about 5 h. The gel was stained with ethidium bromide and the bands were visualised by exposure to UV light.

Result

Twenty six haemophilia A patients were studied from Jan 2000 to Jun 2002. 21 patients were identified as severe haemophilia A, 4 patients as moderate haemophilia A and 1 patient as mild haemophilia A. Inversion of intron 22 were detected in 3 patients (14.3%) out of 21 severe type. Table 1 shows the summary of the results.

Clinical Manifestation	Patients (n)	Inversion intron 22
Severe	21	3 (14.3%)
Moderate	4	Not detected
Mild	1	Not detected

Table 1. Summary of the haemophilia A patients

A specific PCR assay that amplified the factor VIII-intron 22 copy of the Xba 1 polymorphism give a product of 6.2 kb in size. Digestion of the PCR product with Xba 1 resulted in the production of 0.7 kb, 4.8 kb and 0.65 kb fragments in genotypically (+) hemizygous males and 5.4 kb and 0.7 kb fragments in genotypically (-) hemizygous males.

Discussion

From this study intron 22 inversion was detected in 14.3% of severe haemophilia A patients. Inversion intron 22 leads to gross gene rearrangement of factor VIII gene resulting in a division of the gene into 2 halves, facing in opposite directions. This mutation produces a truncated protein product which is unstable, resulting in severe haemophilia A.

The inversion is not found in individuals with moderate and mild form of haemophilia A. The importance of molecular diagnosis in haemophilia A is accurate carrier screening and confirmative diagnosis.

As a conclusion from this study, the inversion mutation of intron 22 can be used as one of the mutation for carrier detection & confirmative diagnosis in haemophilia A patients. For future plan, further studies of other mutations in haemophilia A patients will be continued.

Referrences

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2. Larkish, D., Kazazian, H., Jr, Antonarakis, S.E. & Gitschier, J. (1993) Inversions disrupting the factor VIII gene are a common cause of severe hemophilia A. *Nature Genetics*, 5, 236-241.
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4. El-Maari, O., Oldenburg, J., Caglayan, S. H (1999) Intron 22-specific long PCR for the Xba 1 polymorphism in the factor VIII gene. *British Journal of Haematology*, 105, 1120-1122.
5. De Brasi, C. D., Bowen, D. J., Collins, P. W., Larripa, I. B. (1999) Specific analysis of the intron 22 Xba 1 polymorphism of the human factor VIII gene using long-distance PCR. British Journal of Haematology, 107, 566-568.

Research Presentation
28/11/2002
PPSP, USM

Detection of the Intron 22 Xba1 Polymorphism of the Human Factor VIII Gene Using Long Expand PCR in Haemophilia A Patients

Dr Rapiaah Mustaffa
Assoc Prof Dr Nizam Mohd Isa
Dr Iluni Hayati Ibrahim

Short Term USM Grant
No: 304/PPSP/6131137

Introduction

- Haemophilia A is one of the commonest bleeding disorder in man caused by a deficiency or functional abnormality of coagulation factor VIII.
- Inherited as an X-linked recessive
- Affecting 1:10000 male births world-wide

Classification of The Clinical Manifestation of the disease

Factor VIII activity (%)	Severity of The Disease
<1	Severe
1-5	Moderate
5-30	Mild

Introduction

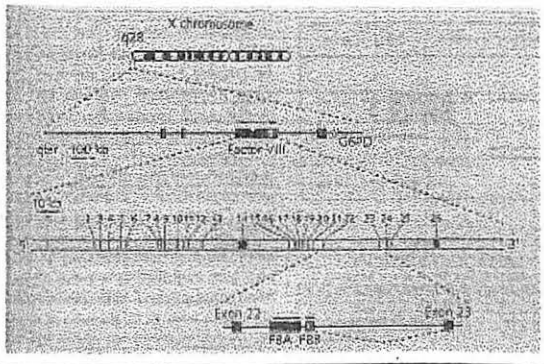
It is caused by mutations in the factor VIII gene.

Factor VIII gene:

- located on the long arm of the X chromosome at Xq28.
- 186 kb in length.
- It consists of 26 exons and 25 introns.

The abnormality of this gene lead to an absence or low level of coagulation factor VIII, causing bleeding problems in the patients.

Factor VIII gene



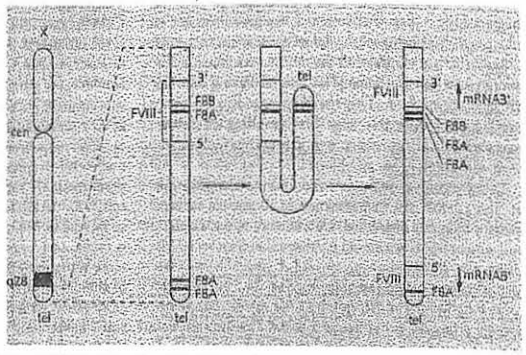
Mutation in factor VIII gene

The mutations found in this disease are heterogenous.

1. Gross gene rearrangements- intron 22 inversion
2. Single base substitutions- 309 different type
3. Sequence deletions- 109 different type
4. Sequence insertions- 28 different type

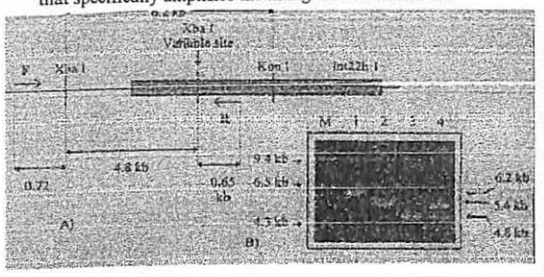
Hot spot mutation: Inversion mutation of intron 22 accounts for about 45% of the severe cases.

Invertion mutation of intron 22



Introduction

The hot spot inversion mutation of intron 22 can be studied by either Southern blot or Long Expand PCR that specifically amplifies the intragenic site intron 22.



Objectives:

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2. To initiate a preliminary work for family/carrier screening in haemophilia A patients.
3. To develop and establish the molecular method for detection of the above mutation.

Materials and Methods

Patient: Haemophilia A patients on follow-up at clinics or admitted in the wards in HUSM, HKB & HKT.

They were diagnosed as patients with haemophilia A

1. Clinically with history of bleeding problems.
2. Routine coagulation screening test including PT, APTT, factor VIII clotting activity (FVIII:C) and vWF:RiCof.

5ml of blood was collected in EDTA container from each patient for molecular analysis.

Materials and Methods

DNA samples: DNA was extracted from peripheral blood leucocytes using standard phenol chlorofom technique with gel separation methods.

Primers: specific for intron 22 of factor VIII gene

Published primer sequences (Caglayan et al, 1999)

Forward primer: 5' GCC CTG CCT GTC CAT TAT ACT GAT GAC ATT ATG CTG AC 3'
Reverse primer: 5' TTC AAC ACG ACC ACC ATC TCT CAA GTC GCC 3'

Material and Methods

Amplification reaction: Long Expand PCR was done in 25 ul total volume containing 200 ng genomic DNA *(Liu. Q. Blood. 1998)*

Reagent	One reaction (ul)
Buffer 2	2.5
7.5 % DMSO	1.87
dGTP	0.63
7-deaza-dGTP	0.63
dNTP (other than dGTP)	1.25
Expand Long Template DNA Polymerase	1.0
50 pmol/ul forward primer	0.5
50 pmol/ul reverse primer	0.5
dH2O	15.15

Materials and Methods

PCR cycle: used GeneAmp 2400 thermocycler (Pekin Elmer)

Temp (°C)	Duration	No of cycles	Remarks
94	2 min	10	Hot start
94	12 sec	10	Denaturation
65	30 sec	10	Anneling
60	7 min	20	Extension
94	12 sec	20	Denaturation
65	30 sec	20	Anneling
60	7 min 20 sec	20	Extension
68	15 min	20	Final extention

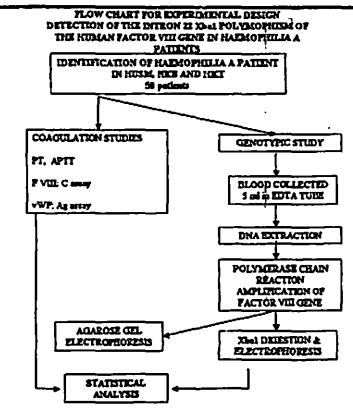
Materials and Methods

Xba1 digestion and electrophoresis:

1. Digestion was done in a total volume of 13 ul containing 10 ul PCR product, 10 units of Xba1 enzyme, 1.3 ul Xba1 Restriction buffer and 0.7 ul H_2O

2. Incubated at 37°C overnight

3. The digestion product was run on 20 cm 1% agarose gel at 100v for 5 hours.



Results

26 Haemophilia A patients were studied from Jan 2000 to Jun 2002.

Clinical manifestation	Patients (n)	Intron 22 inversion
Severe	21	3 (14.3%)
Moderate	4	Not detected
Mild	1	Not detected

Discussion

- From this study intron 22 inversion was detected in 14.3% of severe haemophilia A patients.
- Inversion mutation of intron 22 leads to gross gene rearrangement of factor VIII gene resulting in a division of the gene into 2 halves, facing in opposite directions.
- This mutation produces a truncated protein product which is unstable, resulting in severe haemophilia A.
- The inversion is not found in individuals with moderate and mild form of haemophilia A.

Discussion

- The importance of molecular diagnosis in haemophilia A is for accurate carrier screening and confirmative diagnosis.
- The inversion mutation of intron 22 can be used as one of the mutation screening for carrier detection & confirmative diagnosis in haemophilia A patients.
- Future plan: Further studies of other mutations in Haemophilia A patients will be continued.

Limitation:

Sample size was small.

Acknowledgement:

1. School of Medical Sciences, USM
2. Staff Haematology Department
3. Staff Human Genom Centre
4. Staff HKB
5. Staff HKT